Apoptosis induced by hepatitis B virus X protein in a CCL13-HBx stable cell line

CHAN-YEN KUO[1,3], JU-I TSAI[1], TZU-YU CHOU[1], MAN-JUNG HUNG[3], CHENG-CHUNG WU[4], SHIH-LAN HSU[5] and GUANG-YUH HWANG[1,2]

[1]Department of Life Science, [2]Life Science Research Center, Tunghai University, Taichung; Departments of [3]Obstetrics and Gynecology, [4]Surgery and Education and Research and [5]Education and Research, Taichung Veterans General Hospital, Taichung, Taiwan, R.O.C.

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Abstract. The hepatitis B virus X protein (HBx) critically modulates cell growth by inducing apoptosis or proliferation. We sought to clarify whether HBx-mediated apoptosis in a CCL13 stable cell line (Chang-HBx) with inducible HBx expression proceeds through the extrinsic (death receptor-mediated) and/or intrinsic (mitochondrial-mediated) pathways of apoptosis. We used western blotting, cell viability assays, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, caspase activity assays, JC-1 staining and DNA fragmentation analysis to study the role of HBx in apoptosis. The expression of the pro-apoptotic proteins Bax and Bad and the release of cytochrome c also increased slightly upon HBx induction. JC-1 staining showed a loss of mitochondrial membrane potential upon HBx induction. Additionally, induction of HBx increased the levels of cleaved caspase-9 (intrinsic pathway), caspase-8 (extrinsic pathway) and the common effector caspase-3 as measured by western blotting. This elevation of cleaved caspase-8 or caspase-3 and caspase-9 or caspase-3 decreased in the presence of caspase-8 inhibitor Z-IETD-FMK or caspase-9 inhibitor Z-LEHD-FMK, respectively. Both inhibitors also rescued cell growth, and the caspase-8 inhibitor Z-IETD-FMK prevented apoptotic phenomena including the TUNEL signal. DNA fragmentation analysis showed that these phenomena were not detected in the presence of higher concentration of inhibitors. Our data suggest that HBx induces apoptosis through both extrinsic and intrinsic pathways.

Introduction

Hepatitis B virus X protein (HBx) has been reported to play a critical role in cell growth by regulating cell proliferation and apoptosis (1) and is associated with several signaling cascades (2,3), including Ras/Raf/MAPK (4), JAK/STAT (5) and PKB/Akt pathways (6). However, the effects of HBx on cell growth remain controversial. To elucidate the role of HBx in cell growth, we have created a stable cell line with inducible expression of HBx, CCL13-HBx (Chang-HBx) (7). We have previously reported that HBx inhibited the growth of Chang-HBx cells *in vitro* through the GSK-3β/β-catenin cascade and also inhibited tumor formation *in vivo* in nude mice injected with these cells (7-10). In addition, after Hbx induction, Chang-Hbx cells exhibited the hallmark signs of apoptosis, including propidium iodide (PI) staining, caspase-3 activity, and DNA fragmentation detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (8). However, the mechanism by which HBx mediates apoptosis in these cells remains unclear.

Apoptosis can proceed through 2 signaling pathways: the extrinsic, or death receptor-mediated pathway and the intrinsic, or mitochondria-mediated pathway (11-13). Death receptors are proteins of the tumor necrosis factor (TNF) receptor superfamily, such as the TNF-related apoptosis-inducing ligand (TRAIL) receptors or CD95 (Apo-1/Fas). When death receptors are activated by their ligands, such as TRAIL or CD95 ligand (Fas ligand), they initiate the extrinsic pathway by recruiting the downstream adaptor molecules Fas-associated death domain (FADD) and caspase-8, which aggregate together. This activates caspase-8, which triggers apoptosis by cleaving the downstream effector caspase-3 (14). Mitochondria initiate the intrinsic pathway by releasing cytochrome c, apoptosis-inducing factor (AIF), a second mitochondria-derived activator of caspases/director inhibitor of apoptosis protein (IAP)-binding protein with low PI (Smac/DIABLO), and endonuclease G or Omi/HtrA2 (high-temperature requirement protein A2) (15,16). The release of cytochrome c into the cytosol triggers activation of caspase-3 via formation of the cytochrome c/Apaf-1/caspase-9-apoptosome complex; caspase-3 cleavage, in turn, activates apoptosis. In addition, the integrity of the outer mitochondrial membrane (OMM), which is closely linked to caspase activation, is modulated by the Bcl-2 family proteins, including Bcl-2, Bcl-xL, Bad and Bax (16-18).

Correspondence to: Dr Guang-Yuh Hwang, Department of Life Science, Tunghai University, No. 181, Sec. 3, Chungkang Road, Taichung, Taiwan, R.O.C.
E-mail: gyhwang@thu.edu.tw

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We have previously observed HBx-mediated apoptotic phenotypes in Chang-HBx cells both *in vitro* and *in vivo* (8,10). However, which apoptotic pathway was triggered by HBx in these cells was not clear. Consequently, in this study, we explored the possibility that HBx triggers both the extrinsic and intrinsic pathways of apoptosis. We demonstrated increased expression of caspase-8 and caspase-3 upon HBx induction. In addition, we used Z-IETD-FMK to inhibit caspase-8, a key regulator of the extrinsic apoptotic pathway (11), to study the role of caspase-8 in producing the apoptotic phenotype seen in Chang-HBx cells upon HBx induction. We found that HBx induction elevated cytosolic cytochrome c levels and slightly increased the expression of the intrinsic pathway-associated pro-apoptotic proteins Bax and Bad. HBx also resulted in the loss of mitochondrial membrane potential ($\Delta\Psi m$) observable by JC-1 staining. Moreover, we used the caspase-9 inhibitor Z-LEHD-FMK to confirm the role of caspase-9 in HBx-induced apoptosis. Herein, we propose that HBx induces apoptosis by triggering the caspase-8-mediated extrinsic and caspase-9-mediated intrinsic pathways of apoptosis in the Chang-HBx inducible stable cell line.

Materials and methods

Cell line and cell culture. The CCL13-HBx (Chang-HBx) inducible stable cell line was previously generated in our laboratory (7). Chang-HBx cells were cultured at 37˚C in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1.5 μg/ml tetracycline (Sigma-Aldrich, St. Louis, MO, USA), and penicillin-streptomycin (50 U/ml, Sigma-Aldrich) in a 5% CO_2/95% air atmosphere. The culture medium was replaced every alternate day. For experiments of caspase-8 and caspase-9 inhibition, cells were seeded on 6-well plastic dishes at a concentration of $1x10^5$ per well. Prior to the experiment, 60-70% confluence cells were washed twice with phosphate-buffer saline (PBS), and incubated with serum medium supplemented with various concentrations (0, 0.1, 1, or 5 μM) of caspase-8 inhibitor, Z-IETD-FMK or caspase-9 inhibitor, Z-LEHD-FMK upon HBx induction.

Western blotting. Proteins extracted from the Chang-HBx cells were used for western blotting. The immobilized proteins were detected using anti-HBx (ab235; Abcam Inc., Cambridge, MA, USA), anti-caspase-9 (#9502), anti-caspase-8 (#9746), anti-caspase-3 (#9662; Cell Signaling Technology Inc., Danvers, MA, USA), anti-Bax (#sc-493), anti-Bcl-2 (#sc-7382), anti-Bcl-xL (#sc-643), anti-cytochrome c (#sc-13156; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-Bad (#B-36420; Beckton-Dickinson, Franklin Lakes, NJ, USA), and anti-β actin (#A5316; Sigma-Aldrich). To visualize the proteins, an ECL kit (Millipore Corp., Bedford, MA, USA) was used according to the manufacturer's instructions.

Cell viability. Cells were seeded in a 6-well culture dish at a density of $0.5x10^5$ cell/well and grown in DMEM supplemented with 10% FBS with or without 1.5 μg/ml tetracycline to induce HBx. The culture medium was replaced once every 2 days. The tetracycline-free culture medium was replaced every other day, and the cells were counted until they grew to approximately 60-70% confluence. After counting, the cells were stained with trypan blue to determine viability.

TUNEL staining. Cells were seeded in a 24-well culture plate at a density of $1x10^4$ cells/well and grown in DMEM supplemented with 10% FBS and 1.5 μg/ml tetracycline. Apoptosis-associated DNA fragmentation was visualized with the TUNEL Apoptosis Detection kit (Upstate, Bedford, MA, USA) according to the manufacturer's protocol.

Caspase activity assay. Cells were collected and washed with PBS. The analysis was performed using caspase-3, caspase-8, and caspase-9 Colorimetric Assay kits (Biovision Inc., Mountain View, CA, USA), according to the manufacturer's protocol. The level of caspase activity was detected using a fluorescence microplate reader (Fluoroskan Ascent; Labsystems, Vantaa, Finland).

Mitochondrial membrane potential detection by JC-1 staining. Cells were seeded in 6-well plastic plates at a density of $1x10^5$ per well and allowed to reach 60-70% confluence. Prior to the experiments, the cells were washed twice with serum-free medium. The alterations in mitochondrial membrane potential were assessed using JC-1 Mitochondrial Membrane Potential Detection kit (Biotium Inc., Hayward, CA, USA) according to the manufacturer's protocol.

DNA fragmentation analysis. The cells were collected and washed with PBS. The analysis was performed using the Tissue and Cell Genomic kit (GeneMark, Taiwan), according to the manufacturer's instructions. DNA was extracted using phenol/chloroform and subjected to 1.8% agarose gel electrophoresis.

Results

HBx induction produces hallmarks of apoptosis. When HBx expression was induced in absence of tetracycline, the hallmarks of apoptotic cell death produced by the activities of caspase -9, -8, and -3 were significantly increased (Fig. 1A). HBx induction also increased the expressions of cleaved caspase -9, -8, and -3 (Fig. 1B).

HBx induction produces changes in the mitochondrial membrane potential. The loss of mitochondrial membrane potential ($\Delta\Psi m$) has been considered another hallmark of apoptosis and can be detected by JC-1 staining (19). In healthy cells, JC-1 aggregates in the mitochondria, resulting in areas of red staining known as J-aggregations. In apoptotic cells, JC-1 cannot accumulate in the mitochondria because of disrupted membrane potential and therefore remains in the cytoplasm, where its green fluorescent monomeric form can be visualized by fluorescence microscopy. Upon HBx induction (Fig. 2, right column), more green fluorescence and fewer red J-aggregations in the mitochondria were detected than when HBx was not induced (Fig. 2, left column), demonstrating that the mitochondrial membrane potential was disrupted by apoptosis after HBx induction.

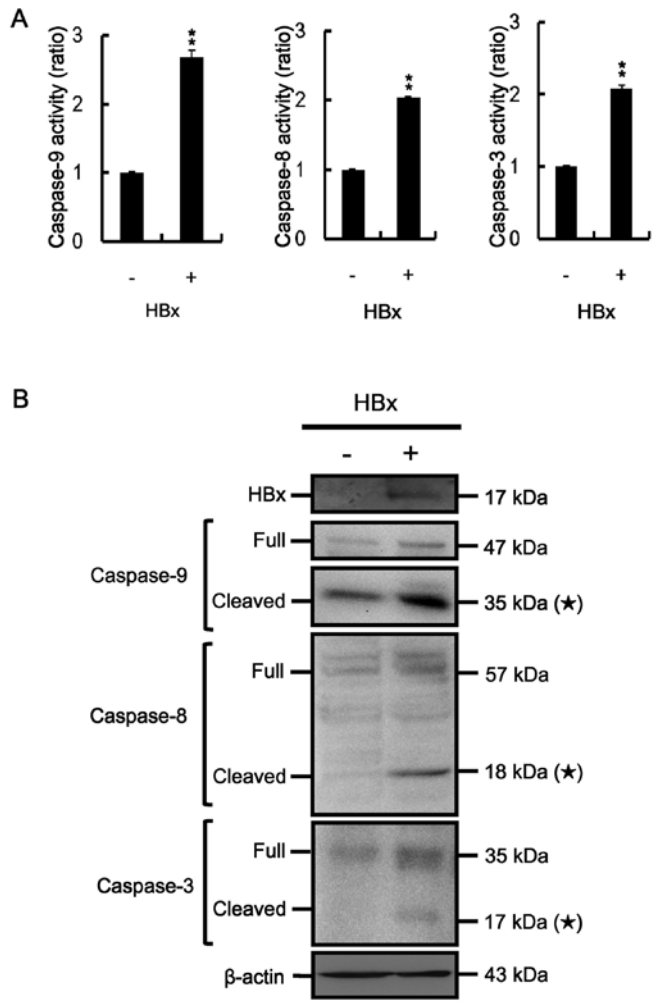

Figure 1. HBx induction increases caspase-9, caspase-8, and caspase-3 activities. (A) Caspase-9, -8 and -3 levels were determined after HBx induction (+) or prior to the induction (-). **P<0.01. (B) Western blot demonstrating changes in the levels of full-length and cleaved caspase-9, -8, and -3 with HBx induction (+) compared to no induction (-). (⋆, indicates cleaved caspase). β-actin was used as a loading control.

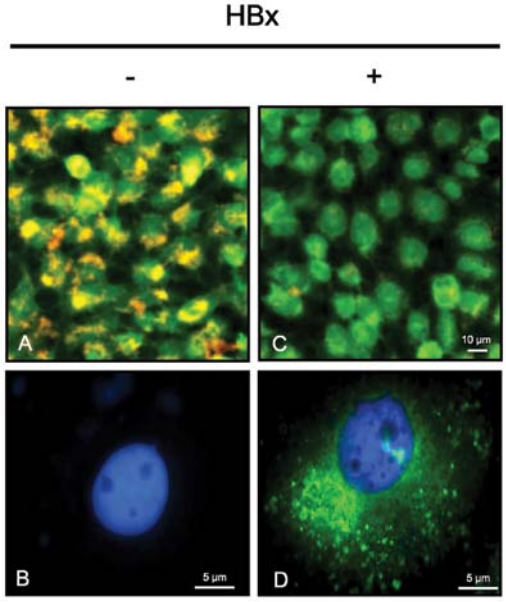

Figure 2. HBx induction decreases mitochondrial membrane potential as assessed by JC-1 staining. In the absence of HBx induction, most cells have abundant red-stained mitochondria, indicative of normal ΔΨm (A and B, left panel). After HBx induction, most cells have abnormally low mitochondrial membrane potential (ΔΨm), indicated by loss of red-stained mitochondria and increased green fluorescence (C and D, right panel). The nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI).

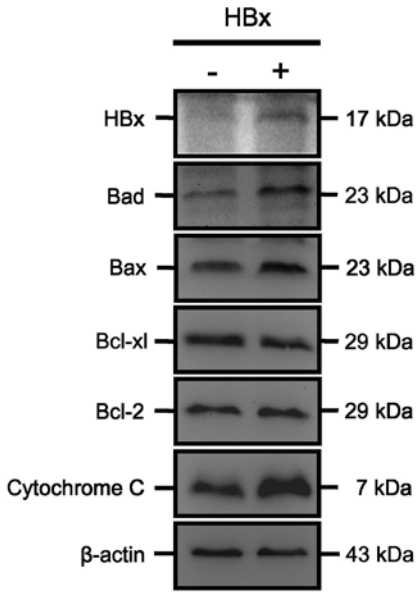

Figure 3. HBx induction alters cellular levels of Bcl-2 family proteins and cytochrome c. HBx induction increased the expression of the pro-apoptotic molecules Bad and Bax and decreased the expression of the anti-apoptotic molecules Bcl-xL and Bcl-2. The expression of cytochrome c was also decreased by HBx induction.

HBx induction affects expression of Bcl-2 family proteins and cytochrome c. It has been reported that the integrity of the outer mitochondrial membrane is mediated by the Bcl-2 family proteins, including Bcl-2, Bcl-xL, Bad and Bax (16-18). We used western blotting to study the effects of HBx on the expression of these proteins. As shown in Fig. 3, HBx induction led to a slight increase in the expression of the pro-apoptotic molecules Bax and Bad. Cytochrome c levels also increased, while the levels of the anti-apoptotic molecules Bcl-xL and Bcl-2 were not altered by HBx induction (Fig. 3).

Caspase-8 is involved in HBx-mediated cell growth suppression. To conclusively demonstrate the involvement of caspase-8 in HBx-induced cell growth suppression, we used western blotting to detect the cellular levels of full-length and cleaved caspase-8 and caspase-3 after HBx induction in the presence or absence of Z-IETD-FMK, a caspase-8 inhibitor. The levels of cleaved caspase-8 and caspase-3 increased with HBx induction, but this increase was blocked by 0.1, 1, or 5 μM Z-IETD-FMK (Fig. 4A). We further investigated the involvement of caspase-8 in HBx-induced cell growth suppression by counting the cell numbers in the presence of 0.1, 1, or 5 μM Z-IETD-FMK (Fig. 4B). Caspase-8 suppressed cell growth when HBx was induced in the absence of Z-IETD-FMK, and 1 and 5 μM Z-IETD-FMK significantly relieved this suppression (Fig. 4C).

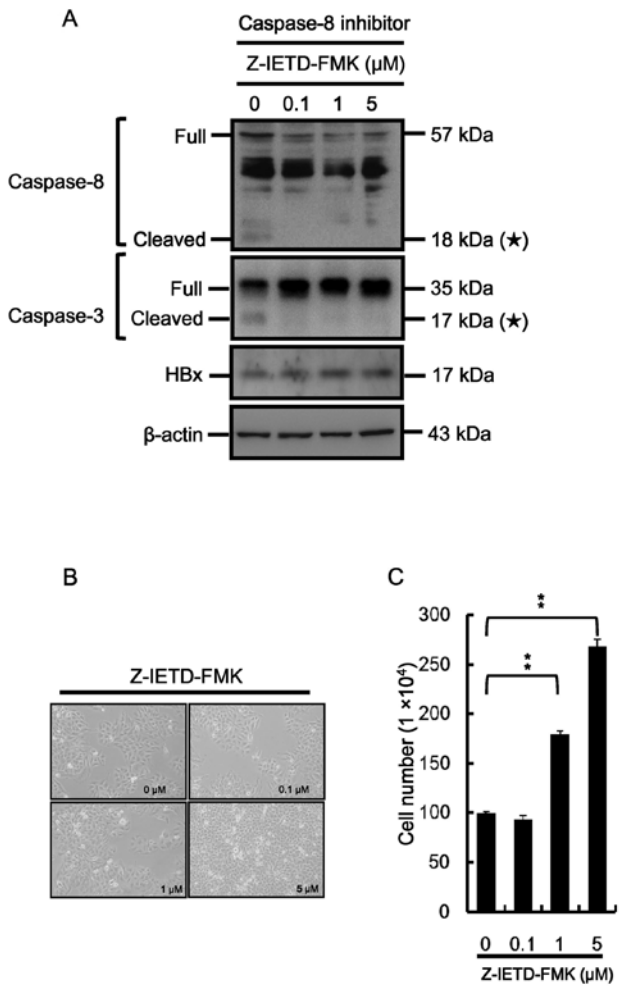

Figure 4. The caspase-8 inhibitor Z-IETD-FMK moderates the HBx-induced cell phenotype. (A) Western blotting showing elevated levels of cleaved caspase-8 and caspase-3 (⋆, active form) observed after HBx induction without the inhibitor. In the presence of Z-IETD-FMK, the levels of cleaved caspase-8 and -3 (⋆) were decreased. (B) Cell morphology was photographed using the phase contrast microscope (Nikon Phase Contrast-2 ELWD 0.3). (C) Cell numbers were significantly increased in the presence of 1 and 5 μM Z-IETD-FMK compared to HBx induction alone. **P<0.01. Data shown are the means of triplicate experiments.

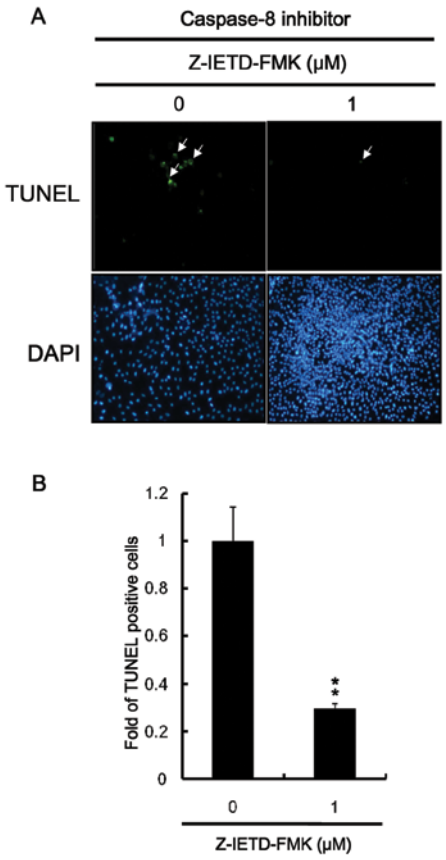

Figure 5. The caspase-8 inhibitor Z-IETD-FMK decreases HBx-induced apoptosis. (A) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining analysis was performed in the absence (left panel) or presence (right panel) of Z-IETD-FMK. Arrows indicate the positive TUNEL staining induced by HBx in the absence of inhibitor. The nuclei were stained with DAPI. (B) Statistical analysis of the relative ratio of TUNEL-positive cells. **P<0.01.

TUNEL staining is a common method for detecting apoptosis (20,21). Results showed that the phenomenon of apoptosis was not observed significantly in the presence of 1 μM Z-IETD-FMK upon HBx induction (Fig. 5). These data suggested that inhibition of caspase-8 by Z-IETD-FMK prevents HBx-mediated apoptosis in Chang-HBx cells.

Caspase-9 is involved in HBx-mediated cell growth reduction. To conclusively determine the effects of caspase-9 in HBx-mediated cell growth reduction, we used western blotting to detect the cellular levels of cleaved caspase-9 and caspase-3 following HBx induction in the presence or absence of Z-LEHD-FMK, a caspase-9 inhibitor. HBx induction increased the levels of cleaved caspase-9 and caspase-3. However, the addition of 1 or 5 μM of Z-LEHD-FMK slightly decreased the levels of these cleaved caspases (Fig. 6A). We then counted cell numbers after HBx induction in the presence or absence of 0.1, 1 or 5 μM Z-LEHD-FMK (Fig. 6B). HBx induction suppressed cell growth in the absence of Z-LEHD-FMK. Z-LEHD-FMK relieved this inhibitory effect, suggesting that caspase-9 is involved in the growth-suppressive mechanism of HBx (Fig. 6C).

Caspase-8 and caspase-9 inhibitors block HBx-induced DNA fragmentation. We specifically evaluated the contribution of the caspase-8 and caspase-9 pathways on the HBx induced cell apoptosis, to verify the results by including the effect of caspase inhibitors on cell apoptosis in presence and absence of HBx induction. Results showed that the phenomena of apoptosis, DNA fragmentation was detected in the presence of 0, 0.1 and 1 μM Z-IETD-FMK (caspase-8 inhibitor) and Z-LEHD-FMK (caspase-9 inhibitor) following HBx induction, but was not detected in the presence of 5 μM Z-IETD-FMK or Z-LEHD-FMK following HBx induction, respectively (Fig. 7).

Discussion

Apoptosis (programmed cell death), plays critical roles in tissue development and homeostasis. Two well-characterized apoptotic pathways are the extrinsic and intrinsic pathways (22). In the extrinsic pathway, stimulation of death receptors such as CD95 (APO/Fas) or TRAIL receptors (TRAIL-R) by CD95

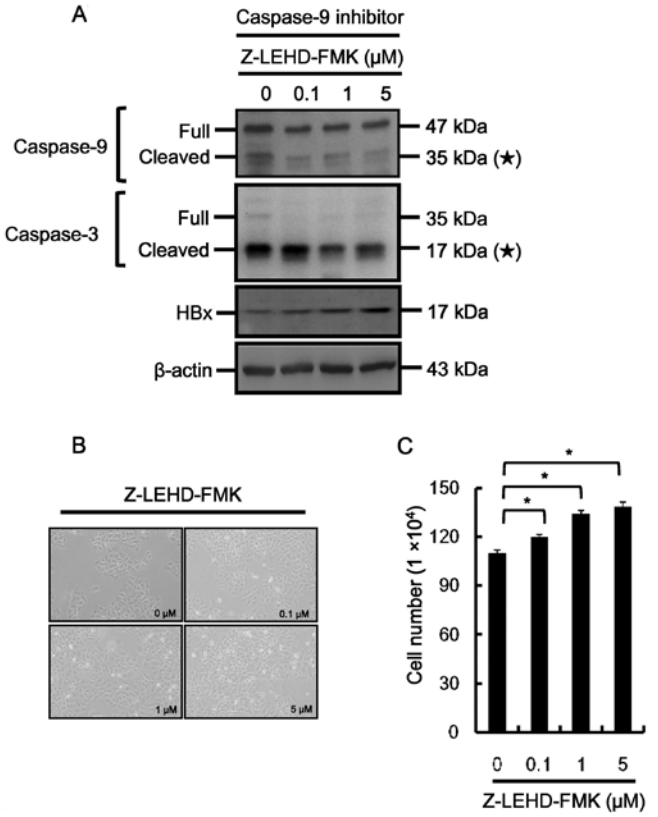

Figure 6. The caspase-9 inhibitor Z-LEHD-FMK also moderates the HBx-induced cell phenotype. (A) Western bloting showing that HBx induction upregulated the levels of cleaved caspase-9 and -3 (⋆, active form) in the absence of inhibitor. In the presence of Z-LEHD-FMK, the levels of cleaved caspase-9 and -3 (⋆) were decreased even after HBx induction. (B) Cell morphology was photographed using a phase-contrast microscope (Nikon Phase Contrast-2 ELWD 0.3). (C) Cell numbers significantly increased in the presence of 0.1, 1 and 5 μM of inhibitor Z-LEHD-FMK compared to HBx induction alone. *P<0.05. Data presented are the means of triplicate experiments.

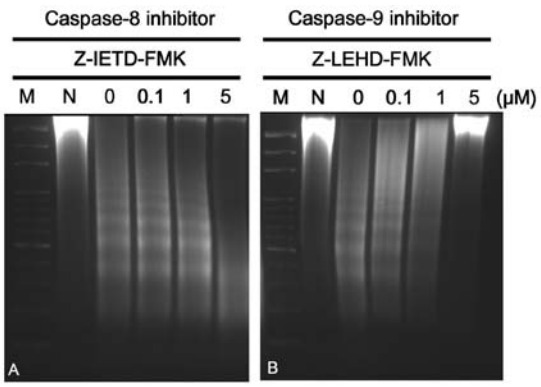

Figure 7. Both inhibitors Z-IETD-FMK and Z-LEHD-FMK moderate the HBx-induced nuclear DNA fragmentation. (A) DNA fragmentation analysis demonstrated that the HBx-sensitized cells underwent apoptosis in absence of or presences at lower concentration (0.1 and 1 μM) of caspase-8 inhibitor, Z-IETD-FMK and (B) caspase-9 inhibitor, Z-LEHD-FMK, however, DNA fragmentation was not detected in the presence of these inhibitors at higher concentration (5 μM). M, molecular weight marker; N, negative control.

ligand (CD95-L) or TRAIL, respectively, results in receptor aggregation and recruitment of FADD and caspase-8. This activates caspase-8, which initiates apoptosis. The intrinsic pathway is activated by the release of cytochrome c, AIF, and Smac/DIABLO from the mitochondrial intermembrane space. The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9 apoptosome complex. Both pathways can involve release of the Bcl-2 family proteins and cytochrome c, which initiates apoptosis (12).

In the present study, we investigated the possibility that HBx induces apoptosis through both the extrinsic and intrinsic pathways. We demonstrated that HBx-induced apoptosis in Chang-HBx cells activates caspase-8, indicating involvement of the extrinsic apoptotic pathway. In addition, the loss of mitochondrial membrane potential detected by JC-1 staining after HBx induction suggested involvement of the intrinsic pathway (Fig. 2). Furthermore, HBx activation slightly increased the expression of the pro-apoptotic molecules Bax and Bad (Fig. 3). Cytochrome c levels also increased, but the levels of the anti-apoptotic proteins Bcl-xL and Bcl-2 were not altered by HBx induction (Fig. 3). We also demonstrated the involvement of the extrinsic pathway-associated caspase-8 in HBx-induced apoptosis, as HBx-induced caspase-8 cleavage and growth inhibition were relieved by the caspase-8 inhibitor Z-IETD-FMK (Figs. 4 and 5). Similar results were observed for the caspase-9 inhibitor Z-LEHD-FMK (Fig. 6), confirming that the intrinsic pathway is also involved in HBx-induced apoptosis. To further confirm the effect of both inhibitors on apoptosis, results showed that DNA fragmentation was not detected in presence of higher concentration of inhibitors (Fig. 7).

HBx proteins are involved in many different signaling pathways, so conflicting results may be due to variations among experimental systems, including different signaling cascades, HBx localization, and HBx expression levels in the cell types used (6,23-28). Cheng *et al* reported that HBx induction led to apoptosis and cell cycle arrest at the G2/M phase through the activation of cyclin B1-CDK1 kinase in Hepa 1-6 (mouse hepatocellular carcinoma cells) or HepG2 (human hepatocellular carcinoma) cells (29). Moreover, Sirma *et al* demonstrated cell cycle arrest at the G1 phase upon HBx induction in Chang-HBx (CCL13) cells (26). Consistent with these reports, many other studies have demonstrated that HBx induces or sensitizes cells to apoptosis (4,8-10,24,28-37); however, there are also reports of apoptosis suppression by HBx (6,38,39). Some reports in which HBx expression was detected by western blotting or immunofluorescence staining indicated that the rate of cell growth was reduced via apoptosis upon HBx induction (26,30,33). Others reported that HBx induction increased cell growth by accelerating proliferation or inhibiting apoptosis, but HBx expression was not demonstrated in these studies (6,38,39). Similar to our findings, Shintani *et al* found that HBx suppressed cell growth or survival in various experimental systems (32).

Several studies suggest that the expression level of HBx is important for hepatitis B virus (HBV) infection (40). HBx levels are low during the early stages of HBV infection, and this low level of expression contributes to transcriptional activation and virus replication by repressing apoptosis. In contrast, high levels of HBV are seen during the late stages of HBV infection. It is possible that higher HBx expression levels contribute to

viral spread by inducing apoptosis (6,24-26,40,41). Our studies support this hypothesis.

Taken together, our data suggest that HBx expression in the Chang-HBx stable inducible cell line induces apoptosis via the extrinsic and intrinsic pathways.

Acknowledgements

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